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Hepatitis B Virus DNA Is Frequently Found in Liver Biopsy Samples From Hepatitis C Virus-Infected Chronic Hepatitis Patients

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Human hepatitis B virus (HBV) and hepatitis C virus (HCV) are two major etiologic agents of chronic hepatitis, which is closely related to the development of hepatocellular carcinoma (HCC). A possible involvement of HBV co-infection was investigated in ongoing HCV-related liver diseases in HCV-infected patients. A prevalence of anti-HBc in anti-HCV–positive/HBsAg-negative chronic hepatitis patients and a low copy number of HBV DNA were found in most of the liver biopsy samples of anti-HCV–positive/HBsAg-negative patients. The present data suggest that HBV co-infects frequently with HCV and may play an important role in the development of HCC in the anti-HCV–positive/HBsAg-negative patients with chronic hepatitis. ***J. Med. Virol. 54: 249–255, 1998.*** © 1998 Wiley-Liss, Inc.

KEY WORDS: HCV infection; HCV-infected hepatitis tissue; HBV co-infection; anti-HBc antibody; HBV DNA

INTRODUCTION

Human hepatitis B virus (HBV) and hepatitis C virus (HCV) are two major etiologic agents of blood-borne hepatitis [Dienstag, 1983; Thaler et al., 1991]. More than 90% of primary liver cancers are related to HBV and/or HCV infection.

Epidemiological similarities between HBV and HCV infections were noted in a report which showed a high prevalence of antibody to HCV (anti-HCV) in blood donors who were positive for antibody to hepatitis B core antigen (anti-HBc) [Gaeta et al., 1990]. Similarities were also noted in blood donors using anti-HBc as a surrogate marker for the prevention of transmission or post-transfusion non-A, non-B hepatitis [Kozoi et al., 1986]. In patients with HBV infection, HBsAg is used as a common denominator of chronic hepatitis, and anti-HBc in the sera of HBV-infected patients implies infection with HBV. Genetic organization and diversity of the HCV genome were well characterized recently [Hijikata et al., 1991; Choo et al., 1991], but HCC-associated markers, which indicate the progression of HCV-induced liver diseases, are still unknown. Moreover, it has not been demonstrated whether the infection of HCV alone can lead to the development of HCC. On the other hand, chronic infection with HBV is closely related to the development of hepatocellular carcinoma (HCC) [Tiollais et al., 1985; Beasley et al., 1981], and the state of HBV DNA in chronic hepatitis and HCC tissues was studied extensively for clarification of the early stage of tumor development. Generally, both closed circular state and random integration of HBV DNA were noted in chronic hepatitis liver tissue, while clonal integration of HBV DNA was present in HCC tissue from HBsAg-positive patient [Koike et al., 1987; Dejean et al., 1984].

One purpose of this study was to assess the prevalence of anti-HBc positivity and the presence of HBV DNA in HCV-infected patients to monitor HBV co-infection. Another was to analyze the etiologic role of each virus in ongoing HCV-related liver diseases in patients co-infected with HBV. A high prevalence of anti-HBc was found in anti-HCV–positive but HBsAg-negative patients with chronic hepatitis. A low copy number of HBV DNA was found in most of the liver biopsy samples from anti-HCV–positive/HBsAg-negative patients with chronic hepatitis carrying HCV RNA in their sera.

MATERIALS AND METHODS

Patients and Serological Markers

As described previously [Koike et al., 1996], serum samples from 4,824 hepatitis patients examined at the Cancer Institute Hospital were subjected to tests for

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HBsAg and anti-HCV. One hundred ninety-nine anti-HCV–positive patients (male, 104; female, 95) (average age: male, 63 ± 10; female, 63 ± 10), thus obtained, were tested for the presence of anti-HBc. Ninety-eight HBsAg-positive patients (male, 42; female, 56) were tested for anti-HCV. Ninety-three anti-HCV–negative/HBsAg-negative patients (male, 32; female, 61) were also tested for the presence of anti-HBc. Among anti-HCV–positive/HBsAg-negative chronic hepatitis patients, 19 patients carrying serum HCV RNA were selected using a reverse transcription–polymerase chain reaction (RT-PCR) assay (HCV Amplicor) (Nippon Roche, Tokyo) for liver biopsy to carry out nested PCR titrating HBV DNA molecules by serial dilution.

Serum HBsAg and anti-HBc were tested by commercial immunoassays (Abbott Laboratories, North Chicago, IL and The Chemo-Sero-Therapeutic Research Institute, Kumamoto). Anti-HCV was detected by a second-generation enzyme-linked immunoassay (Abbott Laboratories). Liver functions were tested according to standard methods (Serdary Research Laboratories, Tokyo).

Titration of HBV DNA Molecules by Serial Dilution

One way to identify a single DNA molecule is to titrate cellular DNA by serial dilution and detection of the DNA molecule from any of the dilution tubes by nested PCR assay.

Each reaction tube contains 45 μl PCR reaction mixture [1X PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl), 200 μM each dNTPs, 0.4 μM each forward and reverse primers, 1.25 units Ex Taq polymerase (TaKaRa)]. One microgram of carrier cellular DNA is added to each tube, except original tube. Sample cellular DNA of 1 μg in 5 μl is added to the original tube and mixed. One-tenth aliquot (5 μl) of the original tube is transferred to the second tube and mixed. One-tenth aliquot (5 μl) of the second tube is transferred to the third tube and mixed. This process is repeated 5 times until a serial dilution of 10^{-5} is obtained. Control huH2-2 DNA, which contains a single copy of HBV DNA in each cell, or human genomic DNA, as a negative control, was similarly diluted. Each reaction mixture thus set up is subjected to PCR assay. After the first PCR reaction, the aliquot of the first PCR product was subjected to the nested PCR reaction using the second primer set to eliminate an artificially amplified product. An aliquot of the nested PCR product was then analyzed by agarose gel electrophoresis, using huH2-2 DNA as a single copy control of HBV DNA. In our present assay condition, a single copy of HBV DNA in huH2-2 DNA can be detected up to 10^{-4}-fold dilution but not in 10^{-5}-fold dilution in the agarose gel. Therefore, the number of HBV DNA in each sample can be estimated by comparing a detected band in corresponding dilution. For example, if the amplified band is detected until 10^{-4} dilution in the gel, the copy number of sample DNA will be one, and if DNA can be seen until 10^{-3}, the copy number of HBV DNA will be 0.1.

DNA Amplification and Blot Hybridization

Cellular DNA was isolated from liver biopsy samples of HCV-infected chronic hepatitis with proteinase K (1 mg/ml) digestion followed by phenol extraction as described previously [Koike et al., 1983]. DNA was similarly prepared by phenol extraction from 200 μl of serum, and ethanol-precipitated DNA was dissolved in 30 μl of distilled water.

PCR was carried out on each sample in 50 μl of a standard reaction mixture (Perkin Elmer-Cetus) that contained 5 μl of 1 μg cellular DNA or 1 μg of serum DNA and 0.4 μM of each primer, for 30 cycles (95°C, 30 sec; 55°C, 40 sec; 72°C, 40 sec) after the sample was denatured at 95°C for 5 min. The primer set to amplify HBs gene was HBS-1/HBS-R1 for the first PCR which yields 310 bp DNA and HBS-11/HBS-R11 for nested PCR with a product size of 241 bp. For X gene amplification, X-Pr/XCD-R1 for the first PCR (product size 441 bp) and XCD-1/XCD-R11 for the nested PCR that yields 236 bp DNA were employed. Primers for the HBc gene were HBC-1/HBC-R1 for the first reaction (product size 307 bp) and HBC-1/HBC-R11 for semi-nested PCR (product size 264 bp). For X gene to HBc gene, XCD-1/HBC-R1 for the first PCR (product size 741 bp) and XCD-2/HBC-R11 for the nested PCR (product size 440 bp) were used. In the X gene to HBc gene region, the lack of continuity of positive and negative strands is present at DR2 (nt 1464–1474) and DR1 (nt 1708–1698), respectively, in relaxed circular molecules of HBV DNA but not in closed circular molecules.

According to Kobayashi and Koike [1984], the sequences of those oligonucleotide primers are as follows:

HBS-1 (nt 43–61) 5′—ATCAGGATTCCTAGGACCC—3′
HBS-R1 (nt 352–333) 5′—AGGACAAACGGGCAACATAC—3′
HBS-11 (nt 77–96) 5′—GCGGGGTTTTTCTTGTTGAC—3′
HBS-R11 (nt 317–297) 5′—GAACCAACAAGAAGATGAGGC—3′
X-Pr (nt 1115–1134) 5′—GAACCTTTGTGGTTCCTCTG—3′
XCD-R1 (nt 1555–1534) 5′—ATTGCTGAGAGTCCAAGAGTCC—3′
XCD-1 (nt 1291–1310) 5′—TCCTTTGTCTACGTCCCGTC—3′
XCD-R11 (nt 1526–1508) 5′—TAAGAGCTTGGGCAAGACC—3′
HBC-1 (nt 1725–1744) 5′—CATGTCCTACTGTTCAAGCC—3′
HBC-R1 (nt 2031–2012) 5′—ACTACTAATTCCCTGGATGC—3′
HBC-R11 (nt 1988–1969) 5′—CCCAGGTGGCCAAATTCATC—3′
XCD-2 (nt 1549–1568) 5′—CAGCAATGTCAACAACCGAC—3′

TABLE I. Serological Markers and HBV DNA of HCV-Infected Chronic Hepatitis Patients*

Patients	Age years	Anti-HCV	Anti-HBc	ALT	Diagnosis	X	HBV DNA[a]		
							HBs	HBc	X-HBc
HH	67	+	+	21	CPH	+	+	–	–
IF	61	+	+	44	CAH2A	+	+	–	–
MTu	81	+	+	31	CAH2A	+	+	–	+
MTi	43	+	+	124	CAH2A	+	+	+	+
TT	67	+	+	149	CAH2A	+	+	+	–
ToT	53	+	+	108	CAH2A	–	+	+	+
TM	48	+	+	34	CPH	+	+	+	–
OT	57	+	+	133	CAH2A	+	+	+	+
SeM	61	+	–	79	CAH2A	+	+	+	+
SH	49	+	–	52	CAH2A	+	+	–	–
HM	58	+	–	101	CPH	+	+	–	–
SN	36	+	–	12	CAH2A	+	+	+	+
OK	24	+	–	63	CPH	+	+	+	–
KK	43	+	–	88	CPH	+	+	+	+
FM	61	+	–	49	CAH2A	+	+	+	–
SaM	61	+	–	24	CAH2A	+	+	+	+
ST	52	+	–	41	CAH2A	–	–	–	–
IT	70	+	–	24	CPH	+	+	–	–
KT	60	+	–	37	CPH	–	+	+	+
AK	62	–	–	28	Normal	–	–	–	–

*All patients are HBsAg negative.
[a]HBV DNA was detected in liver biopsy samples.

As negative controls, PCR buffer without DNA, serum samples from healthy donors without HBV markers, and cellular DNA extracted from λ phage, Raji cells, HeLa cells, placenta, or normal human cells were used. As the one-copy control, the single integrant of HBV DNA in huH2-2 cells was used [Yaginuma et al., 1985]. Southern blot hybridization assay of the amplified DNAs was carried out using the X gene probe (AvaI/EcoRII fragment of 169bp, nt. 1337–1506) and/or the HBs gene probe (HindII/EarI fragment of 191bp, nt. 93–284) [Kobayashi and Koike, 1984].

RESULTS

Anti-HBc in Anti-HCV–Positive and HBsAg-Negative Patients With Chronic Hepatitis

To obtain the frequency of HBV markers in HCV-infected patients, positivities for anti-HBc and HBsAg were examined. As described previously [Koike et al., 1996], anti-HBc–positive patients accounted for 38.2% (average age: male, 66 ± 10; female, 64 ± 11), while HBsAg-positive cases were only 1% of the total anti-HCV–positive patients. Sera collected from anti-HCV–negative/HBsAg-negative patients were also examined for anti-HBc and found in for 18.3% (average age: male, 65 ± 6; female, 63 ± 9). The anti-HBc rate was about 2 times higher in anti-HCV patients than in negative cases. There was no significant bias in age distribution between both cases. The frequency of anti-HBc–positive cases was significantly low in the anti-HCV–negative/HBsAg-negative patients. When anti-HCV was examined using the sera from 98 HBsAg-positive patients, HCV-infection was found to be 5.1% (male, 3; female, 2). This level is about one-eighth that of anti-HBc–positive cases in the HCV-infected patients. In other words, 38.2% of the HCV-infected patients with chronic hepatitis were co-infected with HBV without HBsAg in the sera. HBV co-infection is, thus, detected frequently in HCV-infected patients with chronic hepatitis. As shown in Table I, anti-HBc–positive patients accounted for 42.1% (average age: male, 57.5; female, 60.3) among 19 anti-HCV–positive/HBsAg-negative patients selected for liver biopsy. About 68% of anti-HCV–positive/HBsAg-negative patients have a relatively high titer (>35) of ALT (GPT) level.

HBV DNA in the Cellular DNA From Liver Biopsy Samples of Anti-HCV–Positive and HBsAg-Negative Patients With Chronic Hepatitis

Previously, we examined by Southern blot hybridization the presence of HBV DNA in the HCC tissues, which were derived from non-B HCC patients without HBsAg markers except for the anti-HBc antibody. Integration of HBV DNA [Yaginuma et al., 1985] was detected, similar to the previous reports by Bréchot et al. [1981a,b]. If, in fact, HBV co-infection is involved significantly in HCV-related liver diseases, it should also be possible to detect the HBV DNA in the cellular DNA from liver biopsy samples of HCV-infected patients with chronic hepatitis co-infected with HBV. Cellular DNAs were examined from liver biopsy samples collected from 19 HCV-infected patients with chronic hepatitis and one anti-HCV–negative/HBsAg- and anti-HBc–negative patient (Table I).

In HBV DNA from the HBV–infected HCC tissues, the 3′ end of X gene DNA is located frequently at the virus-cell junction and is able to express its transcriptional activation function as the X-cell fusion gene. On the other hand, the pre-C to HBc gene regions were mostly truncated [Takada and Koike, 1990a,b]. Therefore, the X to HBc gene region could not be amplified by

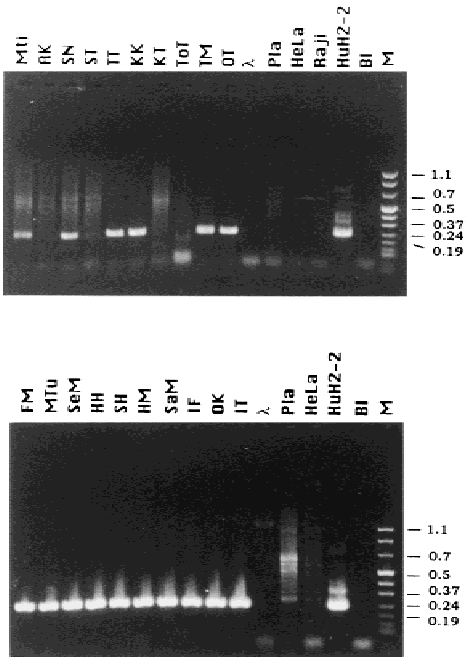

Fig. 1. Detection of HBV DNA in liver biopsy samples from HCV-infected patients with chronic hepatitis. Each 1 μg cellular DNA was subjected to nested PCR assay for X gene, HBs gene, or X gene to the HBc gene region, as described in the text. Mti to OT and FM to IT are sample cellular DNAs from HCV-infected patients with chronic hepatitis. DNA from huH2-2 cell line that contains a single integration of HBV DNA in each cell was used as a positive control. As a negative control, λ phage DNA (λ), human placenta DNA (Pla), and HeLa Cell DNA (HeLa), were used. Bl: no DNA control. M indicates size markers of HpaII, DraI, and HindIII digests of pUCBM21 DNA (Boehringer Mannheim). Primer sets for PCR amplification, X-pr/XCD-R1, and XCD-1/XCD-R11 are described in the text.

the primers (XCD-1 and HBC-R1 for first PCR). If the X gene is present and the HBs gene region as well as the 3′ end of X gene and the pre-C to HBc gene regions (the X-HBc gene region including DR1 and DR2) can be detected, it may be concluded that the HBV DNA in each sample is closed-circular form. Consistent with the previous report [Yang et al., 1996], control reactions showed that relaxed circular DNA molecules were not amplified by the conditions that we used, presumably because of the lack of continuity of both strands at DR1 and DR2 through the region bracketed by the primers for the X gene to the HBc gene. We carried out the nested PCR assay to detect the presence of HBV DNA using each primer set (see materials and methods) for the HBs gene, the X gene, the X-HBc gene, and the HBc gene.

In fact, a 236-bp amplified band, that contained a part of the X gene ORF, was detected in 16 cases among 19 HCV-infected chronic hepatitis DNAs, as shown in Figure 1. For the control experiments, λ phage, huH2-2, human placenta, HeLa cell, Raji cell, and human genomic DNAs were subjected to the same nested PCR assay. The 236-bp amplified band was detected only in huH2-2 DNA, but not in any of the control cellular DNAs. As summarized in Table I, the HBs gene region was detected in 18 cases of HCV-infected patients with chronic hepatitis, and the HBc gene region was detected in 12 cases. Moreover, nested PCR with the primer sets for the X-HBc gene region amplified a 440-bp fragment in nine cases out of 19 chronic hepatitis samples (about 47%). Based on the present results, a significant amount of the HBV DNA may be present as a closed circular form in the HCV-infected chronic hepatitis tissues. We also attempted to detect the presence of closed-circular HBV DNA in the sera from the HCV-infected patients with chronic hepatitis; however, the closed circular form was not detected by nested PCR using the primer sets of XCD-1/HBC-R1 and XCD-2/HBC-R11 (unpublished data).

The copy number of the HBV DNA was examined in 19 DNAs from HCV-infected hepatitis patients with serial dilution using huH2-2 DNA as a control. DNAs were subjected to a nested PCR assay for X and HBs genes, and amplified DNAs were detected by gel electrophoresis. The 236-bp (X gene) or 241-bp (HBs gene) band of HBV DNA was confirmed by Southern blot hybridization using the X gene probe (AvaI/EcoRII fragment of 169-bp) or the HBs gene probe (HindII/EarI fragment of 191-bp) [Kobayashi and Koike, 1984], respectively. Some typical results are shown in Figure 2. As a whole, HBV DNA was detected in 18 cases out of 19 HCV-infected patients with chronic hepatitis as described in Table II. Compared with the single integration in huH2-2 cells, about one copy of X gene and/or HBs DNA at most was detected (sample HM). However, most of the biopsy samples contain less than 0.1 copy of the HBV DNA per cell.

A difference in copy number assay between the X gene and the HBs gene was observed. One extreme case is patient K.T. A possible explanation is that there may be some mismatch in nucleotide sequences between primers and each respective sample used in this study.

DISCUSSION

The prevalence of HBV markers in HCV-infected hepatitis patients and the role of each virus in ongoing liver disease were studied. Surprisingly, anti-HBc was found in about 38.2% of the anti-HCV–positive/HBsAg-negative chronic hepatitis patients, although HCV infection was found in 5.1% of HBsAg-positive patients. On the other hand, anti-HBc positivity was 18.3% in anti-HCV–negative/HBsAg-negative patients. We also detected a low copy number of HBV DNA in about 90% of the anti-HCV–positive, anti-HBc–positive but HBsAg-negative patients with chronic hepatitis.

It is therefore important to determine how or when these anti-HCV–positive patients are infected with HBV, because chronic infections with HBV induce hepatic transformation [Kaklamani et al., 1991; Shirakata et al., 1989; Hohne et al., 1990; Kim et al., 1991]. The chronicity of HBsAg depends primarily on acquisition from mother to infant [Schweitzer et al., 1973], but anti-HBc was increased to 38.2% in anti-HCV–positive/HBsAg-negative chronic hepatitis patients in the present study. There are a number of pos-

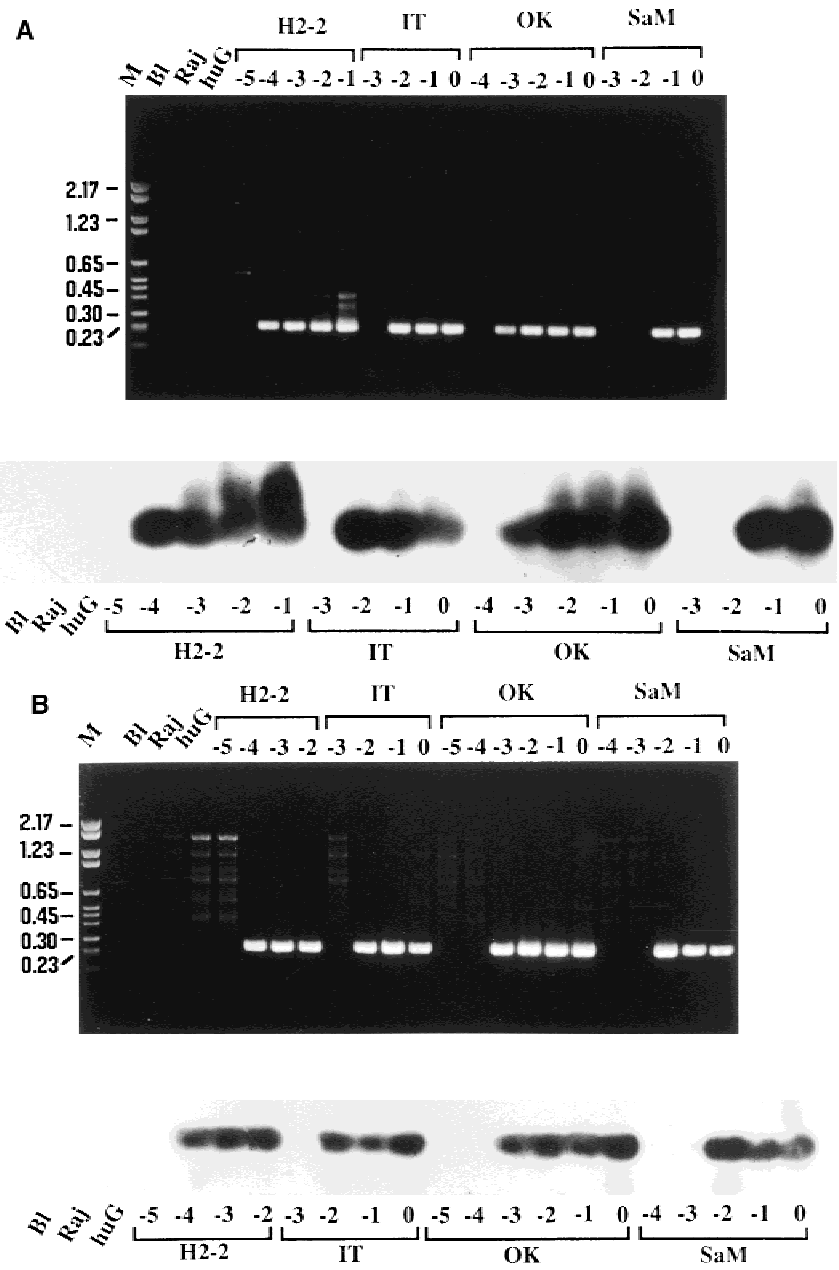

Fig. 2. Copy number assay of HBV DNA in liver biopsy samples from HCV-infected patients with chronic hepatitis. Chronic hepatitis DNAs were subjected to nested PCR assay with serial dilution for X gene **(A)** or HBs gene **(B),** and amplified DNAs were confirmed by Southern blot analysis. SaM to IT is sample cellular DNAs from HCV-infected patients with chronic hepatitis. HuH2-2 DNA was used as a positive control (H2-2), and human genomic DNA (huG), and Raji cell DNA (Raj) were used as a negative control. Bl: no DNA control. M indicates size markers of BglI and HinfI digests of pBR328 DNA (Boehringer Mannheim). Numbers 0 and −1 to −5 indicate 10^n dilution of sample DNA. Primer set for initial amplification was X-pr/XCD-R1 or HBS-1/HBS-R1 and for nested PCR was XCD-1/XCD-R11 or HBS-11/HBS-R11 for X or S gene, respectively. Probe DNA used in Southern blot hybridization is a restriction fragment of 169 bp for X gene or 191 bp for HBs gene, respectively, as described in the text.

sible epidemiological explanations as to why HCV-infected patients would have a high anti-HBc prevalence. One possibility is that if HCV infection may turn off the HBsAg expression and maintain HBV DNA in the host cells by a certain mechanism, HBV DNA can be detected with high frequency among the anti-HCV–positive but HBsAg-negative patients with chronic hepatitis. Anti-HBc among the HCV-related chronic hepatitis patients was double as compared to that of seronegative control patients (38.2% vs. 18.3%) (Table I). In addition, the previous study indicated that anti-HBc among the HCV-infected HCC patients was twice as high as that of HCV-infected chronic hepatitis patients (69.6% vs. 38.2%) [Koike et al., 1996]. Previous and present serological and biochemical findings, which reveal HBV co-infection and the progression of HCV-related liver disease in HCV-infected patients, may imply that many HCV-infected patients are sus-

TABLE II. Copy Number of HBV DNA in Liver Biopsy Samples From HCV-Infected Chronic Hepatitis Patients*

Patients	Anti-HCV	Anti-HBc	Serial Dilution 10^n X gene 0	−1	−2	−3	−4	−5	HBs gene 0	−1	−2	−3	−4	−5
HH	+	+	+	−	−	−	−	−	+	−	−	−	−	−
IF	+	+	+	+	−	−	−	−	+	+	+	−	−	−
MTu	+	+	+	+	+	−	−	−	+	−	−	−	−	−
MTi	+	+	+	−	−	−	−	−	+	+	−	−	−	−
TT	+	+	+	−	−	−	−	−	+	−	−	−	−	−
ToT	+	+	−	−	−	−	−	−	+	−	−	−	−	−
TM	+	+	+	+	−	−	−	−	+	−	−	−	−	−
OT	+	+	+	−	−	−	−	−	+	+	−	−	−	−
SeM	+	−	+	+	+	−	−	−	+	+	+	−	−	−
SH	+	−	+	+	−	−	−	−	+	−	−	−	−	−
HM	+	−	+	+	+	+	+	−	+	+	+	−	−	−
SN	+	−	+	−	−	−	−	−	+	−	−	−	−	−
OK	+	−	+	+	+	+	−	−	+	+	+	+	−	−
KK	+	−	+	−	−	−	−	−	+	−	−	−	−	−
FM	+	−	+	+	+	+	−	−	+	+	−	−	−	−
SaM	+	−	+	+	−	−	−	−	+	+	+	−	−	−
ST	+	−	−	−	−	−	−	−	−	−	−	−	−	−
IT	+	−	+	+	+	−	−	−	+	+	+	−	−	−
KT	+	−	−	−	−	−	−	−	+	+	+	+	−	−
AK	−	−	−	−	−	−	−	−	−	−	−	−	−	−
H2-2		+	+	+	+	+	+	−	+	+	+	+	+	−

*All patients are HBsAg negative.

tained by chronic HBV infection whose HBsAg expression would be turned off. Correlation between anti-HBc and hepatic injury among the anti-HCV–positive patients may be applicable for detecting the high-risk group in terms of HCC.

Since the development of HBV-related HCC is likely induced by the expression of HBV X gene [Shirakata et al., 1989; Hohne et al., 1990; Kim et al., 1991], the escape and clonal growth of the HBV DNA-containing hepatocyte may be easily stimulated or established in the liver of HCV-infected patients. About 47% of anti-HBc–positive but HBsAg-negative HCV-infected patients with chronic hepatitis may have closed circular HBV DNA in addition to the integrated form (Table I), although the copy number is very low and may represent chronic infection with HBV in terms of state of the viral genome [Yang et al., 1996]. The present data suggest that HBV co-infects frequently with HCV and that the presence of low copy number of closed circular as well as integrated HBV DNA per se may have an important role even in stimulation of hepatocarcinogenesis in HCV-infected patients.

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